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Functional microsatellites in cassava (*Manihot esculenta* Crantz): genomic mapping and genetic characterisation in cultivars from Misiones

Microsatélites funcionales en mandioca (*Manihot esculenta* Crantz): mapeo genómico y caracterización genética en cultivares de Misiones

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Abstract
Cassava is a perennial root crop cultivated for human nutrition and livestock feeding. Its roots are consumed peeled and cooked, while the raw roots are processed industrially to obtain starch. In Misiones, farmers have traditionally preferred and preserved several cassava cultivars for specific traits, such as cooking quality, texture of boiled roots, or starch yield. Our objectives were to employ seven EST-SSR primer pairs to map them *in silico* onto the reference genome of *M. esculenta*, and to characterise a group of traditional and new cassava cultivars and lines for Misiones by PCR amplification. Each primer pair mapped uniquely to the expected locus and, for the first time, revealed the corresponding chromosome, gene identity, and primer-mapped region in the reference genome. The functional SSR marker was contained within the region delimited by both primers of each pair. In all 20 studied cassava accessions, these primers produced satisfactory amplification profiles showing bands of expected size. The assayed set of markers yielded a characteristic amplification pattern for each accession, allowing them to be differentiated by this seven-primer combination. This analysis aims to assist the initial steps of genetic characterisation for selection and breeding programs of cassava cultivars in Misiones.

Keywords: Aipim; Cassava; Yuca; Gene markers; Cultivar diversity; NE Argentina.

Resumen
La mandioca es un arbusto perenne cultivado para alimentación humana y del ganado. Sus raíces son consumidas como hortaliza o destinadas a la producción de fécula. En Misiones, tradicionalmente los productores han preferido y preservado varios cultivares según ciertas características, como calidad y textura de cocción, o rinde de fécula. Nuestros objetivos fueron utilizar siete pares de cebadores EST-SSR para localizarlos *in silico* en el genoma de referencia de *M. esculenta*, y caracterizar un grupo de cultivares y líneas tradicionales y nuevas para Misiones mediante amplificación por PCR. Cada par de cebadores mapeó únicamente en el locus esperado, revelando por primera vez el cromosoma correspondiente, el gen asociado y la región blanco de los cebadores en el genoma de referencia. Además, la región delimitada por ambos cebadores de cada par incluyó al respectivo marcador funcional SSR. Los cebadores produjeron perfiles de amplificación satisfactorios en las 20 accesiones estudiadas, con bandas del tamaño esperado. El conjunto de marcadores ensayado produjo un patrón de amplificación característico para cada accesión, permitiendo diferenciarlas con esta combinación de siete marcadores. Este análisis pretende asistir los pasos iniciales de caracterización genética para selección y programas de mejoramiento de mandioca en Misiones.

Palabras clave: Aipim; Mandioca; Yuca; Marcadores génicos; Diversidad de cultivares; NE de Argentina.

Introduction

Cassava (*M. esculenta* Crantz, Euphorbiaceae) is a perennial root crop naturally distributed from the southern United States of America to northern Argentina, and cultivated in tropical and subtropical regions of America, Africa, and Asia for both human nutrition and livestock feeding. This species, also known as *mandioca*, manioc, *aipim*, *macaxeira*, *yuca,* or *cuauhcamotli*, is a major worldwide staple food crop [1, 2]. Its roots are rich in complex carbohydrates and are a valuable source of gluten-free starch. They are consumed peeled and cooked, while the raw roots are processed industrially to obtain starch.

Cooked leaves are often consumed as a vegetable [3, 2].

Significant advances have been made in the genetic and genomic characterisation of this crop to assist selection and breeding programs. In this sense, genetic maps and even a dense composite reference map, comprising thousands of genetic markers, have been constructed [4, 5]. Functional [6] and random [5, 7] SSR markers have also been developed from this species. More recently, several genomic resources have become accessible [8, 4, 9, 10, 11], allowing the characterisation of cassava cultivars, wild accessions, and related species. Moreover, the sequenced, assembled, and annotated genome of the cassava cultivar AM560-2 from Colombia has been designated as a chromosome-level assembly reference genome [12], serving as the most up-to-date unifying platform for cassava genetics and genomics.

As is common worldwide, cassava is mainly cultivated in small-scale farming systems in the provinces of northeastern Argentina. In this region, it is a daily staple used to prepare various traditional dishes, with increasing demand nationwide [2, 13]. In Argentina, Misiones is the leading producer of this crop, and most growers are smallholder families who cultivate it mainly for home consumption but also for commercial purposes, highlighting its economic and sociocultural importance. In this province, cassava production reached 100,000 t in 2024, which was processed exclusively by the starch industry [14].

Traditionally, farmers in Misiones have preserved several cassava cultivars for specific traits, such as cooking quality, texture of boiled roots, and starch yield, to cover the wide range of potential uses. For example, sweet cultivars that are easy to peel and cook are preferred for vegetable consumption, while those with higher starch content are preferred for industrial use [15]. To increase yield and also overcome some issues of traditional cultivars related to biotic stresses, several cultivars and hybrid lines from diverse origins have recently been introduced from the Alliance of Biodiversity International and CIAT (Colombia) through the Clayuca Corporation [16], by the primary starch-producing agricultural cooperatives from Misiones. These cultivars and lines, although introduced for cultivation in NE Argentina, have not yet been included in local trial programs.

Despite the large number of popularly known traditional cultivars and novel accessions available, a comprehensive characterisation of this cassava germplasm remains lacking. Therefore, a depiction of cultivars currently being grown and those that would be brought into cultivation is required for conservation, selection, and breeding purposes [17]. In this regard, molecular markers can provide valuable information on their potential linkage to agronomically interesting genes, genomic context, and genetic variability.

The objectives of this work were i) to localise *in silico* a group of previously developed functional microsatellite markers onto the annotated reference genome of *M. esculenta* and ii) to assay and test their applicability to characterise a group of traditional and new cassava cultivars and lines in Misiones.

Materials and methods

Genomic mapping of functional microsatellite primers: The chromosome-level assembly reference genome GCA_001659605.2 (*M. esculenta*_v8) of cassava cultivar AM560-2 (Colombia) was downloaded from the NCBI Genome Datasets [12]. This genome was employed for *in silico* mapping of a group of seven selected EST-SSR primer pairs (MeESSR8, 15, 19, 22, 23, 28, and 29) developed by Raji et al. [6] (Table 1).

Table 1: Main features of EST-SSR primers mapped in the *M. esculenta* GCA_001659605.2 genome and assayed in traditional cassava cultivars from Misiones and accessions introduced from CIAT.

Marker name	Forward primer/ Reverse primer	Repeat type	Product size (bp)
MeESSR8	ATTGAAATTGGCTTCCGTCA AACCCCCACACCGTACAATA	$TAC(AT)_5T$	166
MeESSR15	TTCGCCTTTCTCATAGCTCAA ATGCATCTGCATGCCTATTT	$(AT)_8$	157
MeESSR19	TTCTCGTCGGCTCCTTTCTA CCCCACTTGATCTGCCTTTA	$(AT)_{10}$	208
MeESSR22	CGACGCATTTTACGTTTTCC CCCCCTCTCATGAATTGAAA	$(CT)C(CT)T(CT)_3CC$	183
MeESSR23	GCTGAGGTTCTGCTGGTTTC CGGAGGATTTCACTGAGGAC	$TT(CT)_4A(TC)_3T$	212
MeESSR28	TTCGATTCAAGAAGGTATTCCA CCTGAAGTACTCGCCTGAGC	$(CT)_{12}$	190
MeESSR29	AATTCTAGGGTCGCCGATTT TGCCATAATAAACCACCTTGG	$(CT)_{11}$	150

The primer pairs were mapped using the Geneious platform (Biomatters Ltd.) with the Geneious mapper at default parameters, except allowing sequence mismatches of 0-10% and a

random number of genomic matches. For each primer pair, we analysed matches to cassava chromosomes, genic loci, gene identity, and the identification of expected simple sequence repeats (SSRs).

Amplification of microsatellite markers in cassava cultivars

Plant material: Seven *M. esculenta* cultivars traditionally grown in Misiones, Argentina, were used. Among them, five cultivars known as *Rocha/Petroski*, *Tacuara/Tacuarita*, *Pomberí/Blanca/Pombero*, *Coloradita/Horquetuda* and *Concepción/Conche* are preferred for both fresh consumption and the starch industry. Additionally, CA 25-1 and IAC-90 are typically intended for industry. In addition, 13 accessions (cultivars and hybrid lines) introduced as a novelty for NE Argentina from CIAT, Colombia, were also considered: PAR 1, PAR 105, BRA 899, BRA 1197, CHN 1, ARG 2, BRA 29, BRA 31, BRA 33, BRA 894, CM321-188, TAI 8, and TAI 16. All these plant materials were maintained in the field and greenhouse at Laharrague Annexe Experimental Field (EEA, INTA, Montecarlo), located in Misiones, Argentina (26°33'36"S 54°40'19"W) (Figure 1).

DNA extraction: Genomic DNA was isolated from 1.5 g of fresh leaves of one individual from each of the 20 cassava cultivars under study, according to Doyle & Doyle [18], with the following modifications. Leaf tissue was ground in a mortar using 4 ml of 2X CTAB isolation buffer. Ground material was poured into a 2 ml tube, and the mortar was rinsed with 4 μl of 2-mercaptoethanol and added to the same tube. 10 μl of proteinase K was added to each sample, followed by a sufficient volume of CTAB buffer to bring the total volume to 2 ml. Samples were incubated at 65 °C with gentle swirling for 15 min and then centrifuged at 3000 rpm for 5 min. About 0.9 ml of the supernatant was recovered and transferred into a new 2 ml tube, and 0.9 ml of chloroform-isoamyl alcohol (24:1, v/v) was added. Samples were then centrifuged at 13000 rpm for 8 min. About 0.9 ml of the supernatant was transferred to a clean tube, and 540 μl of isopropanol was added, gently mixed by hand for about 1 min. Samples were kept at -20 °C for 30 min and then centrifuged at 13000 rpm and 4 °C for 12 min. Finally, the supernatant was gently poured off, and the pellet containing DNA was washed twice with 1 ml of 70% cold ethanol. Finally, the ethanol was discarded, and tubes containing pellets were allowed to dry in a thermal block at 65 °C for 30 min. Then, 100 μL of UP water and 2 μL of RNase were added to each sample. Samples were incubated at 37 °C for 30 min. Sample quality controls were performed by measuring absorbance at 260 and 280 nm in a spectrophotometer and by electrophoresis at 25 V and 1X TBE in 1% agarose gels. DNA samples were stored at -20 °C.

PCR for functional microsatellite amplification: Seven EST-SSR primer pairs (Table 1) previously developed from the *M. esculenta* cultivar TME117 *Isunikankiyan* (Nigeria) [19, 6] were tested in 20 cassava cultivars and lines by PCR amplification. For each cassava cultivar, the amplification reaction was performed using 52.7 ng/μl of template DNA, 1X reaction buffer (PB-L), 5 mM $MgCl_2$, 0.2 μM dNTPs, 0.2 μM of each primer F and R, 0.1 U/μl of Taq DNA polymerase (TAQ Pegasus), and distilled water to a final volume of 20 μl (all final concentrations). Reactions were performed using an Ivema-T21 thermocycler under the following cycle profile: (I) 3-min initial denaturation at 94 °C; (II) 35 cycles of: 30 s at 94 °C, 30 s at 52 °C, 30 s at 72 °C; (III) 5-min final extension at 72 °C.

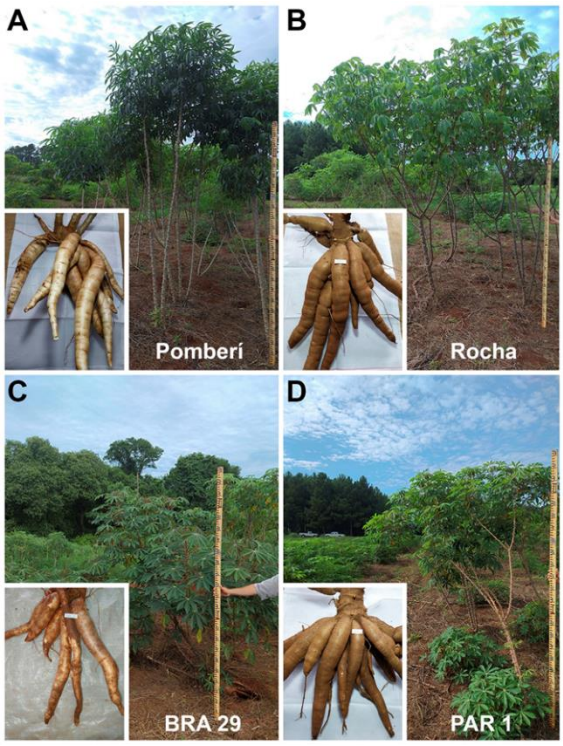

Figure 1: Plants and roots of some *M. esculenta* plant material employed in this work, growing in Montecarlo, Misiones, Argentina. A, B: Misiones' traditional cultivars *Pomberí* and *Rocha*; C, D: line BRA 29 and cultivar PAR 1 imported from CIAT. Note the variability in plant appearance, root number and size, and peel colour.

Polyacrylamide gels for marker visualization: 12% polyacrylamide gels were prepared using 8.871 ml of distilled water, 7.5 g of urea, 1 ml of TBE 5X buffer, 4.5 ml of acrylamide: bisacrylamide (38:2, 40%, w/v) stock solution, 10 μl of N,N,N',N'-tetramethylethylenediamine (temed), and 100 μl of 10% ammonium persulfate (APS) solution. The samples were denatured by formamide treatment at 95 °C for 5 min and then abruptly cooled to 4 °C. Then, 4 μl (containing 1 μl of PCR product and 3 μl of loading buffer) of the denatured samples, and 1 μl of DNA ladder (1 μl PB-L 100 bp DNA ladder, and 3 μl of loading buffer) were loaded onto denaturing 12% polyacrylamide gels. Electrophoresis was carried out at 120 W for 1 h 20 min using a Scie-Plas electrophoresis cell connected to a PowerPac/3000 power supply. After electrophoresis, the gels were fixed in a solution of 10% ethanol and 0.75% acetic acid for 5 minutes. The amplification products were then visualised using silver nitrate according to the Silver Staining-System protocol (Promega, USA), digitised with a Samsung A23 digital camera, and visually analysed.

Results and discussion

Genomic mapping of functional microsatellite primers in cassava

The mapping of seven EST-linked primers to the *M. esculenta* reference genome (GCA_001659605.2) revealed, as expected, seven genomic loci (Figure 2, Table 2). These primers, which were assayed *in silico*, were initially developed by Raji et al. [6] from a set of expressed sequence tags (ESTs) containing microsatellite repeats, obtained from a dehydration-stress transcriptome analysis of TME117, a cassava accession from Nigeria [19]. Since this reference genome represents a consensus of maternal and paternal haplotypes, a unique genomic locus linked to a genic region is expected to be revealed when mapping a primer pair derived from an expressed functional region. The availability of a reference genome sheds light on the target region of these primers, and genomic mapping revealed, for the first time, the corresponding *M. esculenta* chromosome, the gene identity, and the primer-mapped region (mRNA, gene-adjacent genomic region, or ncRNA) for each primer pair. Importantly, each of the seven EST-derived primer pairs was uniquely mapped to the expected genome region, and the region delimited by both primers contained the SSR, in agreement with Raji et al. [6]. (Figure 2).

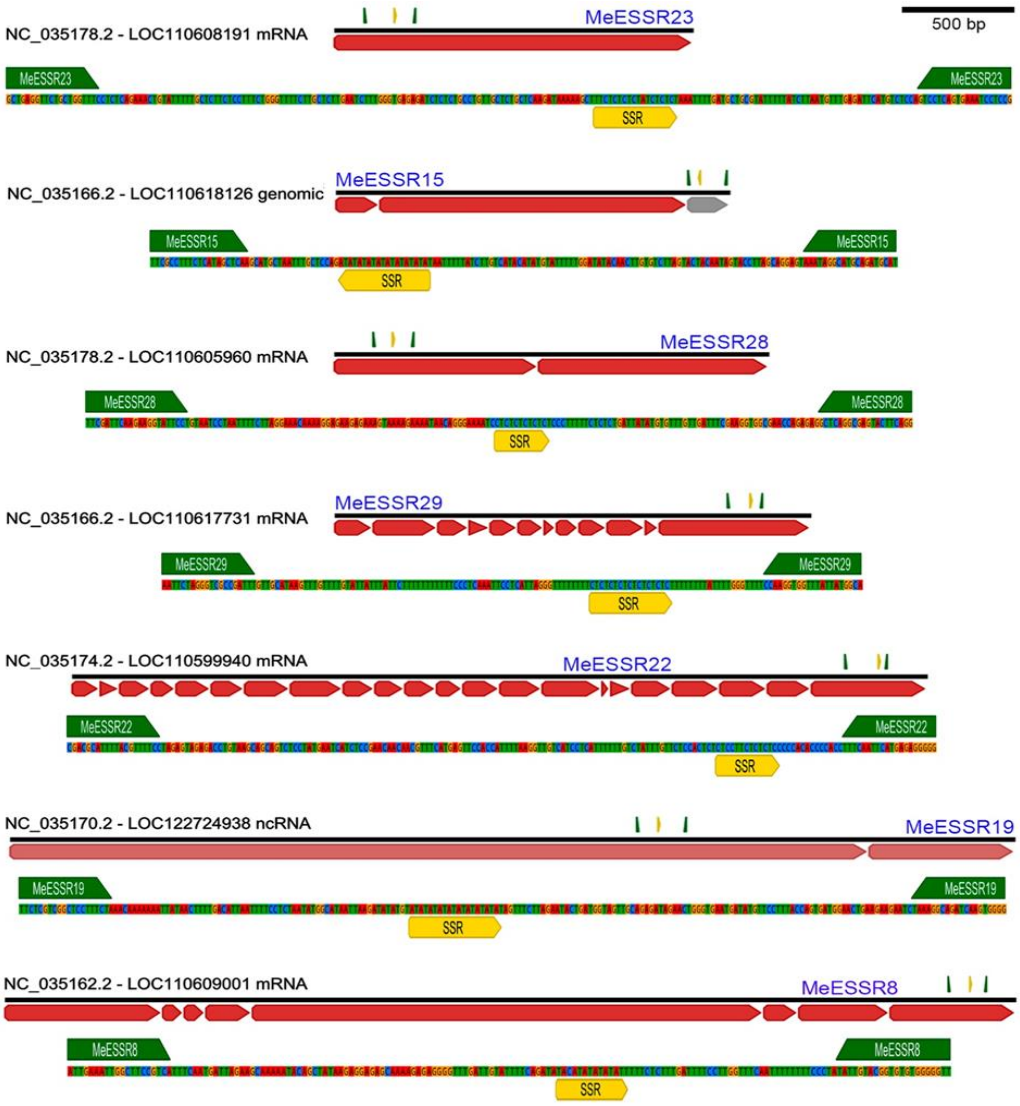

Figure 2: Mapping of EST-linked primers and their products containing SSR markers into the reference genome of *M. esculenta* GCF_001659605.2. Primers were uniquely mapped to expected genome regions encompassing mRNA or adjacent regions, and to ncRNA sequences; NC=chromosome; LOC=gene. Note the detailed amplified region containing SSR markers for each primer pair.

According to the reference genome [12] each primer pair mapped as follows: MeESSR8 to chromosome 2 (NC_035162.2), into the mRNA region of LOC110609001; MeESSR15 to chromosome 6 (NC_035166.2), into the genomic region adjacent to the mRNA of LOC110618126; MeESSR19 to chromosome 10 (NC_035170.2), into an ncRNA of LOC122724938; MeESSR22 to chromosome 14 (NC_035174.2), into the mRNA of LOC110599940; MeESSR23 and MeESSR28 to chromosome 18 (NC_035178.2), into mRNA regions of LOC110608191 and LOC110605960, respectively; and MeESSR29 to chromosome 6 (NC_035166.2), into mRNA of LOC110617731 (Table 2). The gene product encoded by each primer-targeted gene is depicted in Table 2. These results are consistent with the putative functions assigned by Raji et al. [6] through BlastX searches aimed at annotating the respective EST and with gene annotations for coding protein genes from reference genome GCF_001659605.2 via Eggnog-mapper by Aguilera & Grabiele [11] and the NCBI Gene section [20]. Future *in silico* assays of the cassava reference genome that consider functional microsatellite primers not included in this study could reveal their linkage to other agronomically interesting genes and cost-effectively assist selection programs.

Table 2: Summary of seven EST-linked primers *in silico* mapped to the reference genome of *M. esculenta* GCF_001659605.2. The primer target regions are shown relative to the chromosome and gene ID, gene model-related regions, gene products, and the SSR motif contained therein.

Chromosome-Gene ID	Primer-mapped region	Gene product	Primers	SSR motif

NC_035162.2 - LOC110609001	mRNA	PH, RCC1 and FYVE domains containing protein 1	MeESSR8	TACATATATATATT
NC_035166.2 - LOC110618126	Genomic-adjacent to mRNA	chaperone protein dnaJ 49	MeESSR15	ATATATATATATATATATAT
NC_035170.2 - LOC122724938	ncRNA	uncharacterized LOC122724938	MeESSR19	ATATATATATATATATATAT
NC_035174.2 - LOC110599940	mRNA	ATPase 11, plasma membrane-type	MeESSR22	CTCCTTCTCTCTCC
NC_035178.2 - LOC110608191	mRNA	bZIP transcription factor 44	MeESSR23	TTCTCTCTCTATCTCTCT
NC_035178.2 - LOC110605960	mRNA	AP2/ERF transcription factor ABR1	MeESSR28	CTCTCTCTCTCT
NC_035166.2 - LOC110617731	mRNA	chaperone protein dnaJ 15	MeESSR29	CTCTCTCTCTCTCTCTCT

Total DNA extraction and functional microsatellite amplification in cassava

Using the described protocol, total genomic DNA was successfully extracted from all 20 cassava accessions, with concentrations ranging from 377.18 to 707.09 ng/μl (Pomberí and C321-188, respectively).

Amplification reactions using functional microsatellite markers were performed across all 20 cassava cultivars and hybrid lines, including seven traditional from Misiones and 13 newly introduced for field trials in NE Argentina. PCR products were visualised on 12% polyacrylamide gels. A total of seven primer pairs for EST-SSRs were assayed in all samples (Table 1, Figure 3). This same set of primers also successfully mapped to the *M. esculenta* reference genome (Figure 2). In all 20 studied cassava accessions, these primers produced satisfactory amplification profiles, mostly showing clear bands.

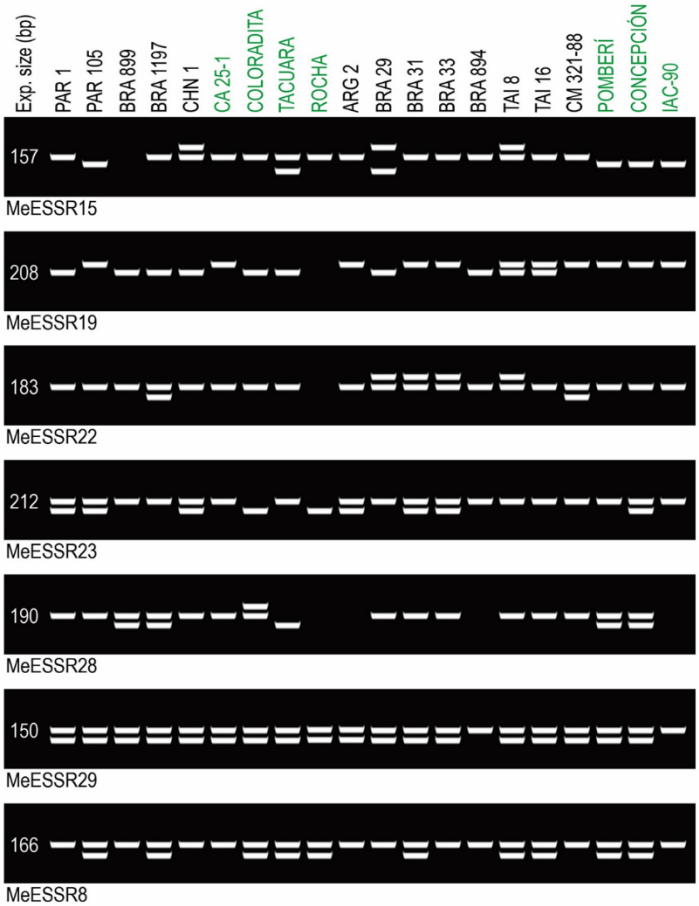

Figure 3: Schematic representation of the EST-linked PCR products assayed in 20 cultivars of *M. esculenta*; in green, traditional cultivars from Misiones. Note the variability in the number and size of amplified DNA bands for each cultivar regarding each pair of primers.

As expected, bands ranging in size from 150 bp (MeESSR29) to 212 bp (MeESSR23) were observed (Figure 3) [6].

Since *M. esculenta* is considered an allotetraploid with disomic inheritance [21, 22] and functional microsatellites are locus-specific [6], the

amplification of one or two bands per locus is expected in each accession. Consistent with this, one or two bands were observed across accessions depending on the primer pair used, thereby indicating a homozygous (one band) or heterozygous (two bands) condition at the respective locus (Figure 3).

Unexpectedly, the traditional cultivar Rocha from Misiones failed to produce many bands for the markers MeESSR19, 22, and 28. Although primers designed to reveal functional markers are from highly conserved regions, slight sequence variations in the target regions, i.e., SNPs, may occur in some cultivars or hybrid lines. Furthermore, if the genotype, *M. esculenta* cultivar TME117 ´Isunikankiyan´ from which these primers were developed, has an SNP/deletion/insertion relative to other members of the species, or if other members have an SNP/insertion/deletion, the corresponding sequence data from those individuals may not align to TME117 [4] and vice versa. Thus, possible sequence differences between the accession TME117 from which the primers were developed and our cultivar *Rocha* from Misiones could explain the lack of amplification products for specific markers in the latter. Different PCR amplification conditions to ensure hybridisation of these primer pairs could help overcome this issue.

Additionally, a different number of total alleles per locus was observed compared to those reported by Raji et al. [6]. In this work, we observed 2, 4, 3, 2, 3, and 2 alleles per locus revealed by markers MeESSSR8, 15, 19, 22, 23, 28, and 29, respectively, while 4, 8, 6, 3, 5, 3, and 5 were previously reported by these authors for the same markers in cassava [6]. Our observations on the number of alleles per locus are coincident with those of Raji et al. [6] only for marker MeESSR28 (3 alleles). These authors developed the primers used and scored them in a group of 24 *M. esculenta* cultivars from Africa, Asia, and Latin America, including landraces, elite breeding lines, putative interspecific hybrids, wild *Manihot* species, and related Euphorbiaceae [6]. Considering the wide range of genotypes, the diversity of accessions we tested here seems to be narrowed by the more limited origins of our material. Most of our studied accessions included cultivars and hybrid lines from Argentina (ARG 2), particularly traditional ones from Misiones, Argentina (*Rocha, Tacuara, Pomberí, Coloradita*, CA 25-1, IAC-90, *Concepción*), Paraguay (PAR 1, PAR 105), Brazil (BRA 899, BRA 1197, BRA 29, BRA 31, BRA 33, BRA 894), Colombia (CM321-188), China (CHN 1), and Thailand (TAI 8, and TAI 16). Thus, most of the plant material we studied represents only a portion of the Latin American diversity of cassava. The lower number of alleles per locus could indicate a constricted diversity of these materials compared to those of Raji et al. [6].

Interestingly, the set of seven assayed markers yielded a characteristic amplification pattern for each of the 20 tested cassava accessions, enabling their differentiation based on this primer combination (Figure 3). Considering the absence of previous variability characterisation experiments for traditional cultivars from Misiones and the need to compare them with new cultivars or lines introduced for cultivation, this type of functional marker may be particularly useful.

By mapping a new set of functional microsatellite primers to the cassava reference genome, it would be feasible to select EST-SSRs linked to other agronomically interesting genes. After accurately selecting an array of interesting and informative primers, this analysis could be extended to other valuable cassava accessions.

Conclusions

Seven EST-SSR primer pairs were mapped *in silico* on the reference genome of *M. esculenta*. Each primer pair mapped uniquely to the expected locus and, for the first time, revealed the corresponding chromosome, the gene identity, and the primer-mapped region (mRNA, genomic-adjacent to a gene model, or ncRNA). As expected, the SSR marker was contained within the area delimited by both primers of each pair.

In all 20 studied cassava accessions, these primers produced satisfactory amplification profiles with bands of the expected sizes. This set of assayed markers yielded a characteristic amplification pattern for each accession, effectively enabling their differentiation through this primer combination.

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